Potential antidepressant-like effects of the biased 5HT1A receptor agonist NLX101 in rats

The Final Work of the Master's degree (TFM) in: Introduction to Research in Mental Health

Instituto de Biomedicina y Biotecnologia de Cantabria (IBBTEC)

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Abbreviations

AMPA – alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF – brain derived neurotrophic factor

CMS – chronic mild stress model

CREB – cAMP response element-binding protein

CSF – cerebrospinal fluid

ERK – extracellular signaling regulated kinase

FST – forced swim test

GABA – gamma aminobutyric acid

GluA1 – glutamate A1 subunit

mTOR – mammalian target of rapamycin

mTORC – mTOR complex

NMDA – N-methyl-D-aspartate

OF – open field test

PET – positron emission tomography

PFC – prefrontal cortex

SSRI – selective serotonin reuptake inhibitor

SNRI – serotonin and noradrenaline reuptake inhibitor

TrkB – tropomyosin receptor kinase B

WB – Western Blot

5-HT – serotonin

Abstract

In recent years, major depressive disorder has been studied from many pathophysiological perspectives and has been concluded as a multifactorial disease with complex interactions between multiple signaling systems. The serotoninergic and noradrenergic systems are implicated in the depression symptoms. In general, antidepressants are created to improve serotoninergic system; however, leading to changes in homeostasis mechanisms. Well known fact that classical antidepressants work only partially and with a delayed onset has triggered the emergence of novel antidepressant agents with supposedly more precise mechanisms of action and less unwanted side effects. They increase serotonin levels in raphe nucleus that can lead to activation of autoreceptors and consequent decrease of serotonin in frontal cortex. A lot of attention has gained serotonin 1A heteroreceptor and its associated mechanisms. NLX101 is a novel serotonin 1A receptor biased agonist that exhibits antidepressant characteristics in animal models. It has shown a functional selectivity for specific G-protein alpha subunits and downstream pathways. Here, we reveal that systemic single dose administration of NLX101 shows antidepressant-like activity in Forced Swim Test, stimulates glutamate and dopamine release in prefrontal cortex dialysate, and increases phosphorylated protein, m-TOR, Glu1A, expression in prefrontal cortex. Overall, NLX101 shows rapid, but not sustained antidepressant-like action at low doses.

Key words: Depression, Serotonin 5HT1A receptor, Behavior, Biased Agonism, Western Blot

Contents

Background

Major depressive disorder (further: depression) is a serious and debilitating mood disorder that can be recurrent and chronic, with specific diagnostic criteria; it requires at least five different symptoms lasting for two weeks and one of them has to be anhedonia or depressed mood (American Psychiatric Association, 2013). It can also be diagnosed by severity using different clinical assessment scales. The symptoms can be classified into somatic (as weight loss or gain) and non-somatic (as decreased concentration or suicidal ideation) (Tolentino and Schmidt, 2018). It is an important cause of years lived with disability and significant contributor to mortality due to suicide. According to the World Health Organization, worldwide, more than 300 million people suffer from this disease (World Health Organization, 2017). About 20% of the treated patients suffer from treatment resistant depression (Jaffe et al., 2019), meaning that at least two antidepressants of the same or different class have not been effective; and only around 30% of the treatment resistant patients achieve remission (Al-harbi, 2012).

Depression is a multifactorial disease with many proposed theories of pathogenesis. For example, gamma-aminobutyric acid (GABA)-ergic deficit, neuroinflammation, immunology (role of cytokines), altered monoaminergic transmission, thyroid and hypothalamus-pituitary axis alterations, glutamatergic hypofunction, and neurogenesis are some of those (Pham and Gardier, 2019). Since the last century 1950s, it has been postulated, that the lack of monoamines (disruption of serotonergic and noradrenergic systems) in the brain is a major cause of the disease (Pytka et al., 2016). Antidepressant treatments have been based on this theory so far. As Adell and Artigas have already

demonstrated, antidepressants preferentially increase the levels of serotonin (5-HT) in raphe nuclei (Adell and Artigas, 1991) and not in the cortex. This theory also does not explain the rapid elevation of 5-HT levels in the neuronal synapsis that contradicts the delayed onset of the antidepressant therapeutic effect or that the lowering of 5-HT in the synapsis trough tryptophane reduction does not induce depression in healthy study participants (Liu et al., 2017). Later, in 1970s other ideas emerged opposing the monoamine theory of the depression. It was proposed that somatodendritic autoreceptor desensitization could lead to increased 5-HT levels; however, 5HT1A receptor antagonists have not been effective as antidepressants in clinical trials (Liu et al., 2017).

In the serotonergic system, from all the receptor subtypes, 5-HT1A receptor seems to play a major role (Kaufman et al., 2016) and has been the most studied one and is involved in the mechanism of action of classical antidepressants. It takes part in diverse central nervous system processes, including cognition, and areas where it can have opposing functions (Newman-Tancredi, 2011). It is found as a presynaptic autoreceptor on soma and dendrites of serotoninergic neurons in the dorsal and medial raphe nuclei. Postsynaptic heteroreceptors are found in the brain areas with serotonergic innervation - limbic system (hippocampus, amygdala, septum) and prefrontal cortex (PFC), mainly on pyramidal cells and GABAergic interneurons (Santana et al., 2004), basically, in the brain regions involved in mood and anxiety (Garcia-Garcia et al., 2014). Some of the 5HT1A receptors have been found in the rat glia as well (Whitaker-Azmitia et al., 1993). These receptors can signal through G-protein dependent and, presumably, G-protein independent ways. 5HT1A receptors are coupled to the inhibitory G protein (Gαi/o) and usually inhibit adenylyl cyclase, thus reducing cyclic adenosine monophosphate (cAMP)

and protein kinase A action (Drago et al., 2008). However, many other signaling pathways have been reported. In the raphe nuclei the autoreceptor activation causes hyperpolarization and $G_{\alpha i3}$ protein coupling, reduction of firing rate, and, consequently, lowering of the extracellular serotonin levels in the projection areas (Garcia-Garcia et al., 2014). Invernizzi et al. also have reported that overload of extracellular 5-HT in the raphe area leads to autoreceptor activation and decrease of 5-HT in the frontal cortex (Invernizzi et al., 1992). Postsynaptic 5HT1A receptors modulate region-specific activity depending on the released 5-HT, they are usually coupled to G_0 and G_{i3} (Newman-Tancredi et al., 2009). They are increased by SSRIs (selective serotonin reuptake inhibitors), tricyclic antidepressants, and electroconvulsive therapy through indirect or direct pathways (Savitz et al., 2009). Therefore, 5HT1A dysfunction can be another possible mechanism involved in depression pathophysiology (Savitz et al., 2009). Autoreceptors create a "short feedback loop" that modulates neurotransmitter synthesis and release (Drago et al., 2008). These receptors are responsible for the delayed effectiveness of antidepressants (SSRIs and selective noradrenaline and serotonin reuptake inhibitors (SNRIs)), which may be solved by the desensitization of 5HT1A autoreceptors leading to the increase of the extracellular 5-HT levels (Dawson et al., 2000). The heteroreceptor activation on pyramidal neurons in PFC also causes hyperpolarization by activating Gαi3 and Gαo proteins (Garcia-Garcia et al., 2014). These receptors can be a potential target for the treatment of neuropsychiatric diseases. 5HT1A receptors are strong modulators of the 5-HT system, since they have different anatomical localization and G-protein coupling populations (Garcia-Garcia et al., 2014). The effects of pharmaceutical targeting may vary depending on the brain area involved; due to this difference in the regional

expression and downstream signaling regulation (Garcia-Garcia et al., 2014) (Newman-Tancredi 2011). Therefore, binding at pre- and postsynaptic sites should be studied and equilibrated to achieve the desired pharmacological effect with minimal adverse events.

Due to the fact that current pharmacological treatment is not always effective and is rather generalized, novel antidepressant agents are emerging. Specifically, glutaminergic system has gained growing attention. Ketamine has shown rapid and sustained (up to 1 week) antidepressant mechanism of action (Zarate et al., 2006). Recently, esketamine, N-methyl-D-aspartate (NMDA) receptor antagonist, in the form of nasal spray in conjunction with an oral antidepressant was approved by the USA Food and Drug Administration for the patients with treatment-resistant depression and depression with suicidal ideations under strict conditions of use and follow-up (SPARVATO ®, prescription information, 2020); however, this drug also has unwanted risks, such as abuse, and psychiatric side effects (Sullivan et al., 2009).

Many preclinical studies with animal models have shown that 5HT1A auto- and heteroreceptors are involved in the pathophysiology of depression (Overstreet et al., 2003) (Lesch and Mössner, 1999). The role of these receptors in depression patients also has been proven in various studies. Postmortem studies regarding suicidal behavior have shown that the levels of this receptor are altered in the brain (Mann et al., 1989), and in vivo PET imaging reports on 5HT1A receptor have shown that availability is reduced in the drug-naïve patients with depression and binding potential in raphe nuclei autoreceptors is higher in medication-free patients that causes less 5-HT release (Hirvonen et al., 2008) (Sullivan et al., 2009). In animal behavioral tests, activation of this receptor at presynaptic site leads to anxiolytic activity, and activation in postsynaptic site-

to antidepressant activity (Newman-Tancredi, 2011). Some attempts in the field to overcome the classical antidepressant challenge have been made, for example, a drug SB-649915-B works as 5-HT transport inhibitor and a 5HT1A/B autoreceptor antagonist increasing extracellular 5-HT in cortex of rats (Hughes et al., 2007). However, it has been concluded that 5HT1A receptor activation in cortex is necessary as well. Clinically tested partial 5HT1A agonists do not show the necessary efficacy and have multiple receptor sites (Newman-Tancredi et al., 2021). Further development has led to discovery of many 5HT1A agonists that couple to these receptors without selectivity, but it has been postulated that unselective activation of these receptors may lead to suboptimal therapeutic effect or even hormonal and cognitive side effects (Depoortère et al. 2019). Based on the fact that different 5HT1A receptor subpopulations are coupled to different G-proteins and therefore activate different signaling pathways, a concept of “biased agonism” has been developed (Newman-Tancredi, 2011).

A novel agent NLX101 (also known as, F15599) works as a biased agonist (Figure 1), meaning it has “functional selectivity.” It has high affinity and selectivity for the 5HT1A heteroreceptor (Newman-Tancredi et al., 2021). It preferentially activates postsynaptic cortical 5HT1A receptors on the GABAergic neurons and not other supopulations of 5HT1A (LLado-Pelfort et al., 2010). The high selectivity of this agonist is based also on the theory that in vivo effects are inhibited by administration of a selective 5HT1A receptor antagonist (Newman-Tancredi et al., 2021). In vitro, NLX101 shows very high activity for phosphorylated extracellular signaling regulated kinase (p-ERK) signal transduction that is similar to other biased agonists as NLX112 or 8-OH-DPAT. However, the observed in vitro results may not translate to activity in vivo (animal experiments) or in neuronal cells,

where it could be different due to possible additional signaling differences (Newman-Tancredi et al., 2021). Other neuronal circuits may also be involved in the signaling that may alter the results.

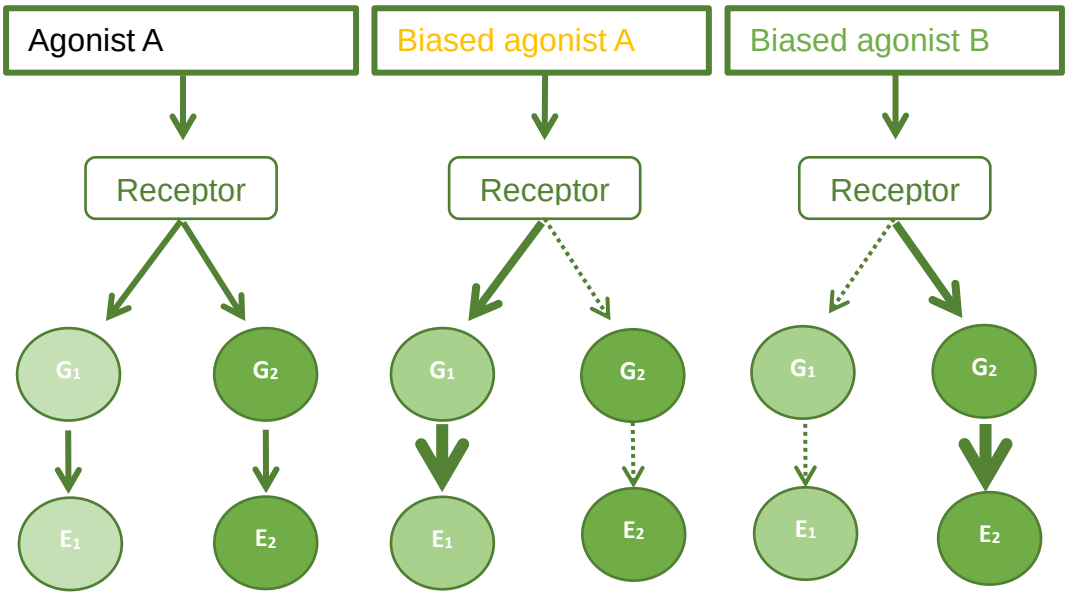

Figure 1. Proposed mechanism of action of the biased agonists. $G_{1/2}$- G proteins; $E_{1/2}$ – coupled effectors

GABAergic inhibitory interneurons in PFC act inhibitory on pyramidal neurons, thus restricting the release of glutamate. However, if the pyramidal neurons are disinhibited via different receptors, they can start firing again. We hypothesize that the novel agent mechanism of action is based on this principle.

The proposed mechanism of action of NLX101 is similar to that of ketamine: the disinhibition of cortical glutamatergic pyramidal neurons via 5HT1A heteroreceptors (NMDA receptors in case of ketamine) that inhibit cortical GABAergic interneurons and GABA release, consequently increasing glutamate release (Newman-Tancredi et al.,

2021). Consequently, glutamate activates postsynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and specific downstream pathways that involve stimulation of rapid (Akt and ERK activation) and long-term effects (brain derived neurotrophic factor: BDNF, regulation); also involving other signaling molecules, as mammalian target of rapamycin (mTOR) (Pham and Gardier, 2019) (Lladó-Pelfort et al., 2010). It has been postulated that NLX101 has a preferential selection for ERK1/2 phosphorylation (Newman-Tancredi, 2011). ERK phosphorylation is necessary for the activation of cascade and subsequent regulation of gene expression trough transcription factors as cAMP response element-binding protein (CREB), and its deficits and thus inactivity in PFC and hippocampus have been associated with pathophysiology of depression in post-mortem brain of suicide subjects (Dwivedi et al., 2001).

Regarding G-proteins, NLX101 shows different potency for G-protein subtypes; it preferentially activates Gαi over Gαo (Newman-Tancredi 2011), mainly activating brain regions involved in mood and cognition. Biased agonists have different interactions with 5HT1A receptor and activation of G-proteins, which leads to involvement of different signaling cascades in the brain neurons (Newman-Tancredi et al., 2021). This, in theory, may improve quickly the depressive symptoms of the patients and reduce unwanted side effects, since it involves particular brain regions.

In the present study, we examined the NLX101 effect on 5HT1A receptors ex vivo, in vivo, and in behavioral paradigms.

Objectives

- The novel agent NLX101 has a potential fast-acting antidepressant effect in the rat depression models.
    - NLX101 might exhibit antidepressant characteristics in behavioral tests as FST and OF after a single dose of systemic administration;
- NLX101 might show changes of neurotransmitter release in neurochemical studies as microdialysis;
- NLX101 might reveal phosphorylated and non-phosphorylated protein (as mTOR, CREB, GluA1, BDNF, ERK) expression changes in molecular studies as WB.
- As a biased agonist NLX101 possibly activates only rat 5HT1A receptors in prefrontal cortex.

Materials and Methods

Animals

Male, 2-3 months old, albino Sprague- Dawley rats were used in this study (Birth date 01/02/21). They were housed in controlled temperature, humidity, and light conditions with free access to the water and food.

Treatment

Animals were injected intraperitoneally F15599, 3-chloro-4-fluorophenyl-(4-fluoro-4{[(5-methyl-pyrimidin-2-ylmethyl)-amino]-methyl}-piperidin-1-yl)-methanone) tosylate salt, (NLX101) (generally supplied by Neurolixis Inc., San Diego, California, USA) 0.16 mg/kg or vehicle (saline) 30 min before the behavioral test, before the tissue excision for Western Blot, or after 3 h stabilization in microdialysis.

All the procedures involving the use of rats and their care were carried out with the previous approval of the Animal Care Committee of the Universidad de Cantabria. All animal protocols were realized in accordance to Spanish legislation and the European Communities Council Directive on “Protection of Animals Used in Experimental and Other Scientific Purposes” (86/609/EEC).

Behavioral Tests

All the tests were performed between 10.00 am and 14.00 pm by experimenters blinded to the treatment. Rats were habituated for at least 30 min before testing. The animals were weighted (300-350 g) before the injection. We used forced swim test (FST) and open field test (OF) for the assessment of the behavior in rats. The data was extracted from program ANY-MAZE and analyzed in MS Excel. The experimental groups were vehicle 6 rats, NLX101 6 rats.

Open-Field Test

The locomotor activity was assessed in the OF test. Animals were placed in 4 open field boxes indirectly illuminated. The floor was covered with with a changeable opaque plastic base. The behavior of thr animals was recorded on a video (ANY maze, Stoelting Europe, Dublin, Ireland). Between each session the field was cleaned with alcohol and feces removed.The time spent in the zones (central, periphery zone) was evaluated in a different study group. This test was used as a screening method to see if there is no significant difference between both study groups and FST would be credible.

Forced Swim Test

Rats were placed in cylindrical tanks with the water temperature of 24±1° C at a level of 30 cm. Three plexyglas cylinders (46 cm height, 20 cm diameter) were prepared and feces counted between each session. We conducted a pretest of 15 min swimming 24 h before the actual test. The FST was conducted 30 mins, 24 h, and 7 days after the injection. On the 1st testing day the animals were injected 0.16 mg/kg NLX101 intraperitoneally 30 mins before the test. Right after the test animals were removed from the tank, dried, and put back in their cages.

The animals were positioned in the pool for 5 mins and their behavior recorded on a video (ANY maze, Stoelting Europe, Dublin, Ireland). Afterwards, we measured the time spent during swimming, immobility, and climbing divided into 5 second periods. The experimenter was blinded to the treatment during the reading of video records.

Molecular studies

Western blot

Western Blot (WB) was conducted to analyze the protein expression and their phosphorylated (active) forms. The animals used in this test were others than the ones used in behavioral paradigms.

The weight of the animals was 250-300 g. The control group received saline (N=6) and the treatment group (N=6) NLX101 0.16 mg/kg (kg x 5ml/1000) injection. Rats were injected with 5-10 min intervals to avoid distress.

The drug injection before sacrifice:

Day 1 – 30 min

Day 2 – 60 min

After sacrificing the animal, the tissue was dissected on the ice. Regions of interest were right and left PFC and right and left hippocampus. Afterwards, the tissue was preserved frozen in -80°C. For further analysis in WB the PFC was used.

Sample preparation

PFCs were weighted and, to gain the cell lysate to extract proteins from adherent cells, samples were homogenized 1:15 with homogenization buffer (10 mM HEPES (pH 7.4), 1.5 mM MgCl2, 100 mM KCl) and 1% protease/phosphatase inhibitor cocktail (Sigma). Lysis buffer was added. The solubilized proteins were collected in the supernatant after centrifugation (14000 RPM, 10 mins, 4°C), put in a fresh tube and placed on ice for the further steps. Samples were prepared in Laemmly buffer (Laemmli Sample Buffer, BIO-RAD, USA) containing beta-mercaptoethanol 5% boiled 100°C for 5 mins for the denaturation of proteins. Aliquotation of proteins with loading buffer and storage of samples at -80°C for further analyzes was done. The protein amount was quantified using the Bradford DC Protein assay, according to manufacturer's protocol (Bio-RAD, USA) We calculated the total protein with reactives (quantification with spectrophotometry, extraction of data in to the program Graphpad and exel, comparison with the pattern graph quantities, gain of data for absorption).

Gel Electrophoresis

In total, 180 µl per sample were collected. We used the supernatants (15 µl per pocket) for the protein separation by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) discontinuous with stacking gel (4 % acrylamide) and in 8.5-15 % separation gels, depending on the molecular weight of each protein, at 100-160V. The electrophoresis was stopped when we observed that molecular weight marker (PageRuler^{TM} Plus Prestained Protein Ladder, 10-250 kDa, ThermoFisher Scientific, USA) reached the limit of the gel. Afterwards, we marked membranes with the number and letter stickers, then the proteins were transferred on nitrocellulose membranes (transfer sandwich) (BioRad, USA) in a wet system 90 mins at 90-100 V (variable amperage) covered with ice. We blocked the membranes for nonspecific fixation of antibodies for 60 mins at room temperature in TBS-T buffer (tris-buffered saline containing 0.1 % Tween 20) with 5% skimmed powdered milk or for phosphorylated proteins - 3% skimmed powdered milk with 200 µl NaV and 100 µl NaF per 100 ml of milk.

Antibody detection

We incubated the membranes in 4-5 ml sealed plastic pockets overnight at 4°C with the primary antibodies (Table 1) diluted in blocking buffer for the detection of proteins CREB, p-CREB, mTOR, p-mTOR, BDNF, GluA1, p-GluA1, ERK, and p-ERK. Afterwards the membranes were washed in TBS-T buffer 3 times 15 mins.

Then we incubated membranes for 60 mins at room temperature in darkness with secondary antibodies at a dilution of 1/15000 (anti-rabbit green anti-mouse red) to afterwards detect the fluorescence. The membranes were washed in TBS-T once again 3 times for 15 mins.

The signal was detected using Odyssey CLx imaging system (LI-COR, USA). We quantified band intensities using densitometric analysis with Image Studio software (LI-COR, USA). The optical densities were standardized to tubulin and protein revels were expressed relative to the amount of vehicle group (100%).

Antigen	Molecular weight (kDa)	Commercial house	Dilution	Host
BDNF	14	ABCAM	1/250	Rabbit
p-GluA1	100	ABCAM	1/250	Rabbit
GluA1	100	ABCAM	1/10000	Rabbit
ERK	42-44	ABCAM	1/1000	Rabbit
p-ERK	42-44	SIGMA	1/200	Mouse
mTOR	289	CELL SIGNALING	1/1000	Rabbit
p-mTOR	289	CELL SIGNALING	1/250	Rabbit
CREB	40	CELL SIGNALING	1/500	Mouse
p-CREB	46	CELL SIGNALING	1/500	Rabbit

Table 1. Primary antibodies as protein markers in Western Blot

Neurochemical studies

Microdialysis

To measure the changes in the extracellular monoamine concentration in the right mPFC after the administration of NLX101, intracerebral in vivo microdialysis was performed in rats. The rat age was 1-2 moths and weight 200-220 g. Five rats were in the control group and 5 in the treatment group. Animals were transformed from their home

cages to the novel environment for microdialysis (transparent acrylic box with sawdust on the floor) for adaptation on the day of surgery.

For the catheter implantation: 4 mm long catheters with semipermeable membranes were prepared in advance. The rats were anesthetized with phenobarbital sodium intraperitoneally 60mg/kg. After the sedative effect was achieved, animals head was positioned according to inter-auricular distance. Section in the skin was made with a bisturi, 4 holes were made to implant 1 catheter and 3 screws for stabilization (AP, DV, L), meninges were perforated for the implantation, probe put in the DV, checked with water, and cement put to seal the catheter. Cement was made from a mixture of resin (TAB 2000) in powder and liquid to polymerize. The coordinates of catheter implantation were calculated according to the Bregma point (AP +3.2, L – 0.6, DV -5.4) and referring to the stereotaxic atlas (Paxinos and Watson, 2005). All the catheters were functional after the implantation and controlled with saline solution.

Awake and freely moving rats, 24 hours after the implantation of probe, during the daytime, underwent: 1) 3 h of stabilization with continuous perfusion of artificial CSF (1.5-1.65 ul/min flow), 2) afterwards each 20 min the dialysate sample collection of 30 µl during 2 h, in total, collecting 6 samples that were used for basal values before the treatment, 3) injection of the NLX101 molecule 0.16 mg/kg intraperitoneally, 4) every 20 min the sample collection, taking 6 samples in total. All the samples were collected in microtubes containing 5 µl of 10 mM perchloric acid and put into the liquid chromatography device (HPLS) overnight. Afterwards levels of dopamine, 5-HT, glutamate, and NA concentration were detected and analyzed in a curve.

Statistical analysis

The statistical analysis was performed using a program GraphPad Prism 5 (GraphPad software, Inc). Experimental groups were compared using unpaired Student's t-test. The microdialysis results were analyzed by ANOVA of repeated measures with treatment and time as factors, with post hoc analysis (Tukey's test). The results were expressed as mean of percentages with the respect to basal levels of neurotransmitters (100%) ±SEM. WB results were analyzed by two-tailed Student's t-test. P-value of <0.05 was defined as statistically significant.

Results

Open Field Test

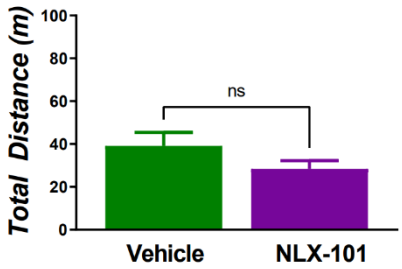

Figure 2. Open field test revealed no significant difference between the vehicle and NLX101

The test was used for the control/reliability of the FST, to analyze whether results of FST are associated with depression-like state and not with general animal activity. Six animals received NLX101 and six – vehicle. No significant difference was found in the results of locomotor activity. (p=0.1799), see Figure 1.

Forced Swim Test

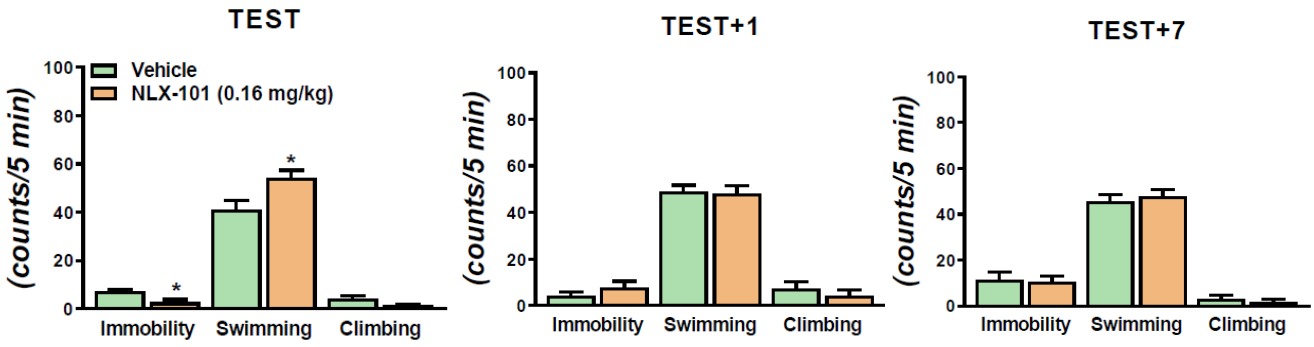

Figure 3. Antidepressant-like effects in the Forced Swim Test. The results 30 mins, 24 h, and 7 days after a single dose of intraperitoneal administration (0.16 mg/kg) revealed that NLX101 decreased immobility and increased swimming compared with control group. *$p<0.05$

During the FST no significant difference between control group and treatment group was found in swimming, climbing, and immobility 24h and 7 days after the treatment with NLX101. However, a significant difference was observed in swimming and immobility on the administration day (30 min before the test). The treatment group showed less immobility and more swimming compared with the control group. Students two-tailed t-test for the results of FST 30 mins showed: Immobility t=3.186, df=8, p=0.0129; swimming t=2.527, df=8, p=0.0354; climbing t=1.902, df=8, p=0.0937 (non-significant). See Figure 2.

Western Blot

No statistical significance was achieved for proteins BDNF (p=0.4177) and pCREB (p=0.1747) after 30 mins of the NLX101 injection. Non-phosphorylated forms of proteins did not reach any statistical significance level neither. However, we observed significant increase in levels of p-mTOR 30 mins after injection and p-Glu1A 60 mins after injection. BDNF results did not reach statistical significance at 30 mins or 60 mins (p =0.0522); however, 2 hours after injection an increase in BDNF level was observed, but this is out of the scope of this paper. Regarding protein ERK, its levels were increased 30 and 60

mins after injection, although, not reaching statistical significance. We can postulate that p-CREB and ERK (15-40 mins) levels raise to significant levels before 30 mins and, afterwards, decline rapidly. See Figure 3.

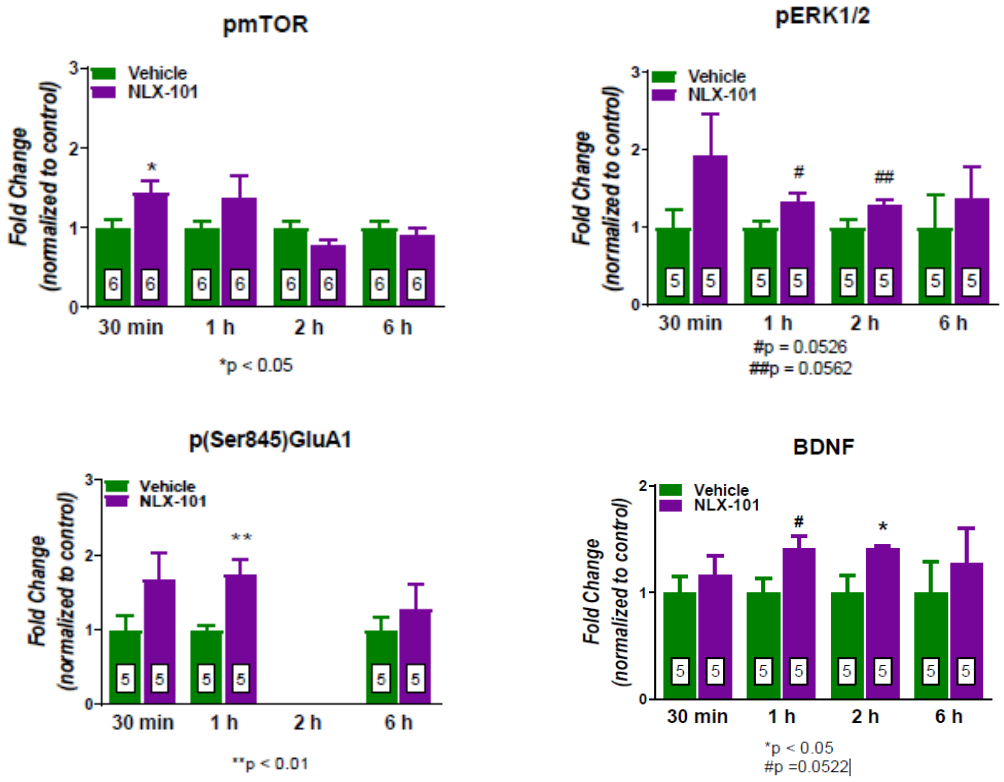

Figure 4. Protein expression after administration of a signle dose of NLX101 0.16 mg/kg intraperitoneally

Microdialysis

First 4 values were obtained to find out basal levels of the neurotransmitters. Results revealed that after the administration of NLX101 during 20 min period increase of approximately 100% vs control was observed in the dopamine$_{ext}$ and 50% vs control

glutamate$_{ext}$ levels; however, no significant change (nor increase, nor decrease) was found in noradrenaline$_{ext}$ and serotonin$_{ext}$ levels. Results on glutamate by two-way ANOVA repeated measures indicated a significant effect of treatment: F(1,12)=7.351; p=0.018908, time: F(9,108)=4.311; p=0.000083, and interaction treatment x time: (9,108)=5.817; p=0,000001. Results on dopamine indicated a significant effect of treatment: F(1,11)= 7.1523; p=0.021624, but not time: F(9,99)= 1.3555; p=0.218785 (non-significant); and a significant effect of interaction treatment x time: (9,99)= 4.3049; p= 0.000096. See Figure 3.

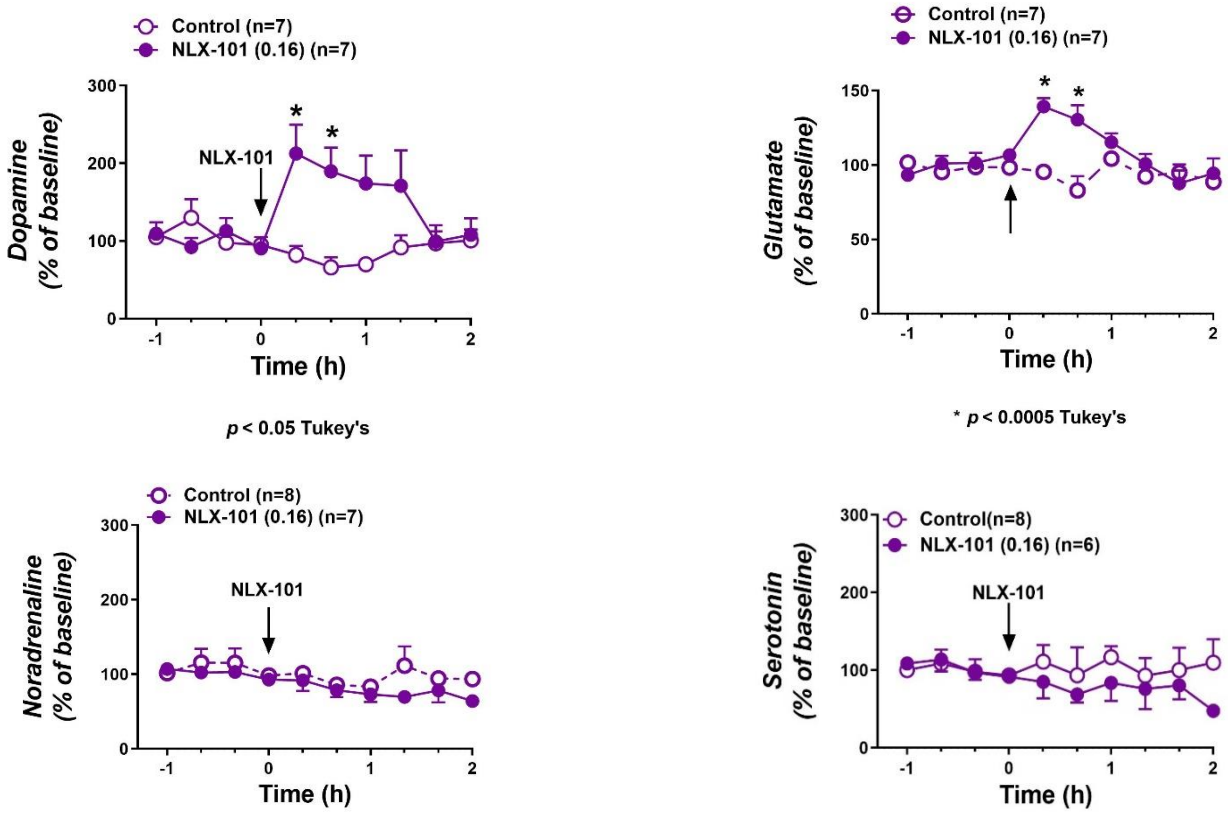

Figure 5. Release of the monoamine neurotransmitters in the mPFC after the systemic administration of NLX101 (0.16 mg/kg i/p). NLX101 stimulated the release of dopamine$_{ext}$ and glutamate$_{ext}$ in dialysate. The results are expressed as the mean of percentages +- SEM in respect to the basal levels. The arrow indicates the injection of NLX101. *p<0.05*; **p<0.0005*. posy hoc Tukey's test with ANOVA repeated measures. Number of animals in the parentheses.

Discussion

We intended to find potential antidepressant characteristics of the novel agent NLX101 in rats in the current study. Newman-Tancredi has reported that in rats NLX101 exhibits potent antidepressant-like activity in the FST (Newman-Tancredi, 2011). It is widely accepted that FST is sensitive to activation of cortical 5HT1A heteroreceptors (Newman-Tancredi et al., 2021). In our behavioral examinations FST results revealed decreased immobility and increased swimming time on the administration day (30 minutes). That could indicate the rapid effects of NLX101; however, short-term (24 hours after injection) and long-term (sustained effect of ketamine is 7 days) effects should be assessed and chronic stress models studied, to find out and conclude whether this molecule can have sustained effects. Also, chronic mild stress (CMS) model should be considered, since it has higher translational potential for mental disease studies, Depoortère et al. reported that in CMS NLX101 showed efficacy with a rapid antidepressant-like effect and that the effects were sustained during 2-week treatment and continued 4 weeks after stopping the therapy (Depoortère et al. 2019).

Assie et al. have reported high antidepressant activity in acute and repeated administration of NLX101 to rats in FST by completely eliminating immobility. Moreover, they reported that NLX101 effects are continued with sustained reduction in immobility for at least 8 h (Assie et al. 2010). In our case, the immobility was significantly decreased only on the day of administration and no effect was seen 24 h or 7 days after the injection, we did not measure the effect 8 h after the administration. They also propose that CMS model should be used complementary to the FST.

In our microdialysis study NLX101 triggered the release of dopamine and glutamate in the rat PFC at the dose 0.16 mg/kg intraperitoneally. We did not study the dialysate of Raphe nuclei or ventral hippocampus (5-HT); therefore, it is not possible to comment directly on the autoreceptor involvement. However, no reduction in 5-HT levels was observed, which would indicate that the autoreceptors were not activated (Hjorth and Sharp, 1991), we can also assume from this that 5HT1A heteroreceptors on pyramidal neurons were not activated. Newman-Tancredi has reported that in microdialysis tests NLX101 modestly activates 5HT1A autoreceptors at higher dose than the one necessary to activate dopamine release in rat mPFC and that the chronically administered dose necessary to desensitize autoreceptors is very high (20 mg/kg for 14 days), which might give us an idea that this agonist has minimal effect on them (Newman-Tancredi, 2011). As Lladó-Pelfort and colleagues have already reported, NLX101 increases the dopamine levels in PFC, the effect that was significantly reversed by WAY100635, selective 5HT1A antagonist (Lladó-Pelfort et al., 2010). This is associated with 5HT1A heteroreceptor activation. Dopamine levels were elevated in our dialysate of the PFC. Most probably, NLX101 activated mesocortical dopaminergic neurons in the Ventral Tegmental Area via projections from pyramidal neurons in mPFC (Diaz-Mataix et al., 2005). Glutamate levels were significantly elevated plausibly trough disinhibition of GABAergic interneurons in the mPFC. Compared to a study with systemic administration of ketamine, where glutamate, noradrenaline, and 5-HT were elevated (Lopez-Gil et al., 2019), we found that 5-HT levels were not changed, which might give a clue that NLX101 has a short-term effect compared to ketamine. Our microdialysis data suggest that NLX101 preferentially activates postsynaptic 5HT1A heteroreceptors over presynaptic.

NLX101 at low doses stimulates rat medial PFC (mPFC) pyramidal neurons and dopamine release. It does not activate autoreceptors at low doses (Newman-Tancredi, 2011). The 5HT1A biased agonist activity in different regions was proven by neuronal electric activity as well. The effects at auto- or heteroreceptor site can be distinguished by the firing rate of dorsal Raphe nucleus 5-HT neurons or of pyramidal neurons in cortex. NLX101 shows a dose-dependent inhibition of Raphe neuron firing and an increased firing of cortical pyramidal neurons at low doses, favorably activating pyramidal neurons (Llado-Pelfort et al., 2010).

Newman-Tancredi and colleagues have reported that in ELISA analysis NLX101, 30 mins before sacrifice, shows modest inhibition of p-ERK in hippocampus and, at a same dose, significantly stimulates p-ERK in the frontal cortex, compared with other 5HT1A receptor biased agonists, as NLX112, that strongly inhibited p-ERK in the hippocampus at low dose, suggesting its preference for 5HT1A autoreceptors. The proposal that NLX101 prefers to activate heteroreceptors is also based on the fact that it stimulates c-Fos expression in the frontal cortex rather than in raphe nucleus (Newman-Tancredi et al., 2021). It has been postulated that NLX101 elevates ERK phosphorylation, compared with cAMP reduction, in the rat PFC, which is controlled by postsynaptic 5HT1A receptors and inhibits this pathway in hippocampus at similar doses (Newman-Tancredi et al., 2009). In our study, p-ERK levels elevated at 30 and 60 mins, but not significantly. Newman-Tancredi has also proposed that the elevation in BDNF and ERK protein levels is responsible for the rapid action ox NLX101; however, we would like to propose that its rapid effects could be based on p-mTOR and p-GLUA1 increase in expression. Since BDNF involved in rapid processes can be the one from the reserves in vesicles (activity

dependent release) and not involving downstream pathways. In contrast, for the sustained antidepressant-like activity of ketamine, early activation of BDNF release and its binding to TrkB receptors in the hippocampus is necessary. (Pham and Gardier, 2019).

We studied protein mTOR and its phosphorylated form. Our results revealed increased expression of p-mTOR. This is consistent with previous studies of ketamine mechanisms. Li and colleagues have reported that ketamine rapidly activates mTOR signaling pathway, which leads to increase in synaptic signaling proteins and, subsequently, increase in new spine synapses (Li et al., 2010). mTOR is phosphorylated trough activity-dependent fast release of BDNF and subsequent activation of TrkB and its downstream pathways PI3K-Akt and MEK-ERK. Phospho-mTOR further increases local translation of the proteins as GluA1 (Duman et al., 2012).

We also studied GluA1 and its phosphorylated form expression after administration of NLX101, since, to the best of our knowledge, no publications exist regarding this matter. GluA1 is one of the ionotropic glutamate receptor subunits that, together with other subunits, form a calcium-permeable AMPA receptor and trigger its integration into post-synaptic membrane (Zhang and Abdullah, 2013). It has an important role in the synaptic plasticity. The increased expression and phosphorylation at serine^{831} or serine^{845} site of GluA1 subunit in the cortex and hippocampus is a known mechanism for the treatment of depression (Kiselycznyk et al., 2013).

There were some limitations to this study. First, the number of animals could have been higher to gain statistical power. Second, there could be some bias in the analysis of FST, since it is a subjective evaluation based on the experience of evaluator. Third, since my work in the laboratory was one month long, some research was out of the scope of this

paper: we did not use other, higher doses of administration that might lead to activation of other receptor sites or lead to longer, more lasting effects; we could also study the effects of direct intracortical administration of NLX101 and compare them with system administration; we did not study the GABA release in dialysate. Forth, no correlation analyses were conducted, since we used different animals for behavioral and molecular tests; therefore, we can only conclude associations between the results. Currently, the work on this molecule is still in progress. It is therefore crucial to investigate in further studies the NLX101 preference to activate 5HT1A receptors on the GABAergic interneurons and not on the pyramidal neurons in the PFC, where this receptor has inhibitory role, theoretically inducing opposite effects to the activation of 5HT1A on GABA interneurons.

Conclusions

Single administration of NLX101 of 0.16 mg/kg systemically displayed rapid (30 min), but neither sustained (up to 24 h) neither long-term (up to 7 days), antidepressant-like effect in the forced swim test. Systemic (i/p) administration of NLX101 increases the efflux of dopamine and glutamate, but not noradrenaline nor serotonin in microdialysis study. Therefore, we can speculate that NLX101 works by previously mentioned theory of GABAergic interneuron disinhibition. NLX101 produced significant rise in p-mTOR protein expression 30 mins after the injection; in Glu1A protein expression 60 mins after injection. A rise in p-ERK levels was observed in WB of 30 and 60 mins; however, not reaching statistical significance, we assumed that its activation time is very short (less than 30

mins). Overall, NLX101 shows rapid, but not lasting synaptic actions at low doses. Possibly due to the activation of synaptic plasticity signaling pathways in the mPFC via p-mTOR (activating mTORC1) and p-GluA1 (a major subunit of AMPA receptor) protein expression, and not preferentially via p-ERK and BDNF (since BDNF is activity-dependent release) pathway as it has been presented in previous studies. Further investigation in rodents should be conducted, for example, with repeated dosing and in long-term or using chronic stress models. The NLX101 effects should be studied via translational methods between species to further develop it in the clinical setting for patients with neuropsychiatric diseases.

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